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Biosynthesis of ethyl caffeate via caffeoyl-CoA acyltransferase expression in *Escherichia coli*

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Abstract

Hydroxycinnamic acids (HCs) are natural compounds that form conjugates with diverse compounds in nature. Ethyl caffeate (EC) is a conjugate of caffeic acid (an HC) and ethanol. It has been found in several plants, including *Prunus yedoensis*, *Polygonum amplexicaule*, and *Ligularia fischeri*. Although it exhibits anticancer, anti-inflammatory, and antifibrotic activities, its biosynthetic pathway in plants still remains unknown. This study aimed to design an EC synthesis pathway and clone genes relevant to the same. Genes involved in the caffeic acid synthesis pathway (tyrosine ammonia-lyase (TAL) and *p*-coumaric acid hydroxylase (HpaBC)) were introduced into *Escherichia coli* along with 4-coumaroyl CoA ligase (4CL) and acyltransferases (AtCAT) cloned from *Arabidopsis thaliana*. In presence of ethanol, *E. coli* harboring the above genes successfully synthesized EC. Providing more tyrosine through the overexpression of shikimate-pathway gene-module construct and using *E. coli* mutant enhanced EC yield; approximately 116.7 mg/L EC could be synthesized in the process. Synthesis of four more alkyl caffeates was confirmed in this study; these might potentially possess novel biological properties, which would require further investigation.

Keywords: Acyltransferase, Alkyl caffeate, Hydroxycinnamic acid conjugate, Metabolic engineering

Introduction

Hydroxycinnamic acids (HCs) are abundant in nature. They are synthesized via phenylpropanoid pathway in plants and serve as the building blocks for other phenolic compounds, such as flavonoids, coumarin, lignin, proanthocyanidins, cutin, and suberin [1]. HCs form conjugates with other molecules as well. The conjugate of caffeic acid (an HC) and quinic acid is called chlorogenic acid [2]. Avenanthramides are amides formed from HCs and anthranilate derivatives [3]. Besides these, conjugates of HCs with other compounds (spermine, puterine, tyramine, dopamine, tryptamine, and glycine) have also been reported [4, 5].

Ethyl caffeate (EC) is a conjugate of caffeic acid and ethanol, and is found in some plants, namely *Prunus yedoensis* [6], *Polygonum amplexicaule* [7], and *Ligularia fischeri* [8]. In particular, *Ligularia fischeri* is grown in eastern Asia and is used in herbal medicine. The biological effects of *L. fischeri* are derived from EC. EC is known to exhibit anticancer [8], anti-inflammatory [9], and antifibrotic activities [10]. In addition, it has the potential to regulate blood pressure by inhibiting aldosterone synthase [11].

The synthetic pathway for EC in plants has not been fully elucidated yet. Caffeic acid is synthesized from tyrosine by deamination and hydroxylation, and needs to be activated by the attachment of coenzyme A (CoA). Although high-molecular-weight alcohols, such as C18, C20, and C22 alkan-1-ols, have been reported to be synthesized from long-chain fatty acids in plants [12], how the ethyl group is provided during the synthesis of EC still remains a mystery. Ethanol is presumably synthesized in hypoxic condition [13], which is also suitable for the synthesis of EC; however, other ethyl group donors may

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also be present. The mechanism underlying the conjugation of caffeoyl-CoA with ethyl group donor still remains unknown. Previous study on the biosynthesis of suberin might provide a clue to the process of generating an EC conjugate. Suberin is an ester of long-chain primary alcohols and HCs [14]. The conjugate reaction is mediated by aliphatic suberin feruloyl transferase in *Arabidopsis thaliana* [15]. Therefore, an enzyme of this family might catalyze the conjugation reaction to form EC, although the origin of ethyl group in plants still remains unknown.

Microbial systems (mainly *Escherichia coli* and *Saccharomyces cerevisiae*) have been used to synthesize phytochemicals [16]. Among the variety of phytochemicals, phenolic compounds are mostly synthesized in microbial systems, since the biological synthetic genes have been characterized in many plants and hosts are engineered to provide more substrates [17]. Introduction of caffeic acid synthesis pathway genes into *E. coli* and engineering of the latter to provide more tyrosine resulted in the successful synthesis of caffeic acid. Tyrosine-overexpressing *E. coli* strain, due to overexpression of $tyrA^{fbr}$, *ppsA*, *tktA*, and $aroG^{fbr}$ and deletion of *pheA*, *tyrA,* and/or *tyrR* genes, was employed for the reaction, and TAL (Tyrosine ammonia-lyase) from either *Rhodotorula glutinis* or *Saccharothrix espanaensis* and *p*-coumaric acid hydroxylase genes (HpaBC from *E. coli* or Sam5 from *S. espanaensis*) were introduced into it. The final titers of caffeic acid, reported in various publications, are quite different; one report used tyrosine-overproducing *E. coli* by introducing $tyrA^{fbr}$, *ppsA*, *tktA*, and $aroG^{fbr}$ while deleting *pheLA* and *tyrA* genes, and overexpressing TAL. Tyrosine from *R. glutinis*, together with HpaBC, enabled 766.7 mg/L titer of caffeic acid production [18]. Another report introduced *TAL* from *S. espanaensis* and *Sam5* into tyrosine-overproducing *E. coli* strain, in which *tyrR* was disrupted and $tyrA^{fbr}$ and $aroG^{fbr}$ were overexpressed. The strain synthesized 150 mg/L caffeic acid [19]. In this article, we reported the successful synthesis of EC in *E. coli*. We selected a gene encoding a protein that would synthesize EC from caffeoyl-CoA and ethanol (Fig. 1). Subsequently, we manipulated the shikimate pathway in *E. coli* to increase the synthesis of caffeic acid. By introducing genes for the conjugation of caffeic acid and ethanol into an engineered *E. coli* strain, and providing ethanol, we could successfully synthesize EC.

Materials and methods

Constructs

Caffeoyl-CoA transferases from *A. thaliana* (*AtCAT1* [AT5G63560.1] and *AtCAT2* [At5g41040]) were cloned using reverse transcription-polymerase chain reaction (RT-PCR). The primers were as follows: aaacatATGGCC GACTCATTCG and aaggtaccTCATATATCCATAAT CCCTTGGA for *AtCAT1*, and aaacatATGGTTGCT GAGAACAATAA and aaggtaccTTATATCTGTAAAAA CTGTTCTTGA for *AtCAT2*. The restriction enzyme recognition sites (NdeI and KpnI) are underlined in the primer sequences. The PCR product was sequenced to verify the nucleotide sequence. The previously cloned *Os4CL* [20] was subcloned into EcoRI and HindIII sites of pCDF-duet1 (Novagen) and the resulting construct was named pC-OS4CL. *AtCAT1* and *AtCAT2* were subcloned into NdeI/KpnI site of pC-OS4CL, respectively, and became pC-OS4CL-AtCAT1 and pC-OS4CL-AtCAT2, accordingly. The previously cloned *SeTAL* and *HpaBC* [21, 22] were subcloned into EcoRI and HindIII sites and NdeI and XhoI sites of pET-duet1 (Novagen), respectively, resulting in the construct named pE-SeTAL-HpaBC.

Synthesis of EC

Escherichia coli BL21 (DE3) transformant, containing pC-OS4CL-AtCAT1 (B-EC1 in Table 1), was grown in Luria–Bertani (LB) broth with 50 μg/mL spectinomycin overnight at 37 °C. The culture was inoculated into fresh LB medium and grown at 37 °C until the OD_{600} reached 1.0. Thereafter, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to a final concentration of 1 mM and the cells were incubated at 18 °C for 16 h. The amount of cells, corresponding to OD_{600} of 3 in 1 mL, was harvested and resuspended in 1 mL of M9 medium containing 1% yeast extract, 2% glucose, 50 μg/mL spectinomycin, and 1 mM IPTG. Caffeic acid and ethanol were also added to the medium resulting in a final concentration of 100μM and 1%, respectively. The culture was incubated at 30 °C for 24 h. The reaction

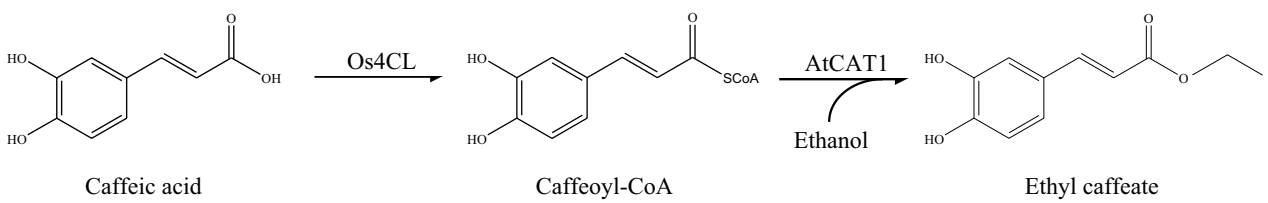

Fig. 1 Scheme for the synthesis of ethyl caffeate from caffeic acid

Table 1 Plasmids and *Escherichia coli* strains used in this study

Plasmids and *E. coli* strains	Relevant properties or genetic markers	Source
Plasmids		
pCDFDuet	CDF ori, Sm^r	Novagen
pETDuet	F1 ori, Amp^r	Novagen
pACYCDuet	P15A ori, Cm^r	Novagen
pC-OS4CL	pCDFDuet carrying *4CL* from *Oryza sativa*	This study
pC-OS4CL-AtCAT1	pCDFDuet carrying *4CL* from *Oryza sativa* and *CAT* from *Arabidopsis thaliana*	This study
pC-OS4CL-AtCAT2	pCDFDuet carrying *4CL* from *Oryza sativa* and *CAT* from *Arabidopsis thaliana*	This study
pE-SeTAL-HpaBC	pETDuet carrying *TAL* from *Saccharothrix espanaensis* and *HpaBC* from *Escherichia coli*	This study
pA-aroG-tyrA	pACYCDuet carrying *aroG* and *tyrA* from *E. coli*	[21]
pA-$aroG^f$-$tyrA^f$	pACYCDuet carrying $aroG^f$ and $tyrA^f$ from *E. coli*	[21]
pA-$aroG^f$-ppsA-tktA-$tyrA^f$	pACYCDuet carrying $aroG^f$, *ppsA*, *tktA*, and $tyrA^f$ from *E. coli*	[21]
pA-aroL-$aroG^f$-ppsA-tktA-$tyrA^f$	pACYCDuet carrying *aroL*, $aroG^f$, *ppsA*, *tktA*, and $tyrA^f$ from *E. coli*	[30]
pA-aroL-aroE-aroD-aroB-$aroG^f$-ppsA-tktA-$tyrA^f$	pACYCDuet carrying *aroL*, *aroE*, *aroD*, *aroB*, $aroG^f$, *ppsA*, *tktA*, and $tyrA^f$ from *E. coli*	[28]
pA-aroC-aroA-aroL-aroE-aroD-aroB-$aroG^f$-ppsA-tktA-$tyrA^f$	pACYCDuet carrying *aroC*, *aroA*, *aroL*, *aroE*, *aroD*, *aroB*, $aroG^f$, *ppsA*, *tktA*, and $tyrA^f$ from *E. coli*	[31]
Strains		
BL21 (DE3)	F- *ompT hsdSB(rB- mB-) gal dcm lon* (DE3)	Novagen
BT	BL21(DE3) *FRT-ΔtyrR::FRT-*kan^R *-FRT*	[21]
BTP	BL21(DE3) *ΔtyrR::FRT- ΔPheA::FRT-*kan^R *-FRT*	[21]
B-EC1	BL21 harboring pC-OS4CL-AtCAT1	This study
B-EC2	BL21 harboring pC-OS4CL-AtCAT2	This study
B-EC3	BL21 harboring pC-OS4CL-AtCAT1 and pE-SeTAL-HpaBC	This study
B-EC4	BL21 harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-tyrA-aroG	This study
B-EC5	BL21 harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-$tyrA^f$-$aroG^f$	This study
B-EC6	BL21 harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-$aroG^f$-ppsA-tktA-$tyrA^f$	This study
B-EC7	BL21 harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-aroL-$aroG^f$-ppsA-tktA-$tyrA^f$	This study
B-EC8	BL21 harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-aroL-aroE-aroD-aroB-$aroG^f$-ppsA-tktA-$tyrA^f$	This study
B-EC9	BL21 harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-$tyrA^f$-aroC-aroA-aroL-aroE-aroD-aroB-$aroG^f$-ppsA-tktA	This study
BT-EC7	BT harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-aroL-$aroG^f$-ppsA-tktA-$tyrA^f$	This study
BTP-EC7	BTP harboring pC-OS4CL-AtCAT1, pE-SeTAL-HpaBC, and pA-aroL-$aroG^f$-ppsA-tktA-$tyrA^f$	This study

product was extracted by ethyl acetate and dried in speed vacuum. The sample was eventually dissolved in dimethyl sulfoxide (DMSO) and analyzed by high-performance liquid chromatography (HPLC).

Analysis of EC

The reaction product was purified using thin layer chromatography (TLC; TLC silica gel 60 F254; Millipore, Burlington, MA, USA). The mobile phase was a mixture of dichloromethane, ethyl acetate, and formic acid (9:1:0.25). The structure was determined using NMR [23]; ^{1}H NMR of ethyl caffeate (400 MHz, Methanol-d4): δ 1.31 (3H, t, J = 7.1 Hz), 4.21 (2H, q, J = 7.1 Hz), 6.25 (1H, d, J = 15.9 Hz), 6.78 (1H, d, J = 8.3 Hz), 6.94 (1H, dd, J = 8.3, 1.9 Hz), 7.03 (1H, dd, J = 1.9 Hz), and 7.53 (1H, d, J = 15.9 Hz).

HPLC analysis of the product was performed as earlier [24].

Results and discussion

Screening of genes for EC synthesis

EC is an ester of caffeic acid and ethanol. Plants contain genes that encode an enzyme capable of forming alkyl hydroxycinnamate ester [25, 26]. These enzymes have been involved in suberin and cutin biosynthesis, and are known to use long-chain alcohol (i.e. dodecan-1-ol) as an alkyl group donor. We assumed these enzymes to possibly use low-molecular-weight alcohol. Two genes (*AtCAT1* and *AtCAT2*), which encoded fatty alcohol

caffeoyl-coenzyme A acyltransferase [15, 26], were cloned and tested for the production of EC. Either of *AtCAT1* and *AtCAT2* was subcloned, along with *Os4CL* encoding 4-coumarate CoA ligase, since caffeic acid needed to be activated. *E. coli* harboring either pC-OS4CL-AtCAT1 (B-EC1) or pC-OS4CL-AtCAT2 (B-EC2) were fed caffeic acid and ethanol. The formation of EC in the culture filtrate was examined using HPLC. Formation of a new product was observed in both culture filtrates. While the *E. coli* strain B-EC1 converted all the caffeic acid into the reaction product, the other strain (B-EC2) still had unreacted caffeic acid. This suggested that *AtCAT1* had better catalytic efficiency than *AtCAT2*. The reaction product from the strain B-EC1 was purified, and was identified as EC by NMR. The results indicated that *AtCAT1* could conjugate caffeoyl-CoA with ethanol to make EC (Fig. 2).

We further tested other low-molecular-weight alcohols as substrates. *E. coli* strain B-EC1 was fed caffeic acid and various low-molecular-weight alcohols, including methanol, ethanol, propanol, butanol, and pentanol. Analysis of the supernatant showed that all the alcohols tested were substrates for the synthesis of corresponding alkyl caffeates (data not shown). Propanol turned out to be the best substrate among all alcohols tested, based on the unreacted caffeic acid remaining. The different permeability of each alcohol into the *E. coli*, and other factors, such as volatility, were found to have an influence on the titer of each alkyl caffeate. Together, the results indicated that different alkyl caffeates could be synthesized by providing different alcohols. We did not explore the alcohols with more than six carbon atoms, since the solubility of such alcohols would be significantly low (in case of hexanol, the water solubility was 5.9 g/L).

We investigated the maximum amount of EC synthesized when *E. coli* was fed 1% ethanol. *E. coli* harboring pC-OS4CL-AtCAT1 (B-EC1) was fed different concentrations of caffeic acid (0.5, 0.7, 1.0, 1.2, 1.5, and 1.8 mM) and 1% ethanol. Caffeic acid was completely converted into EC up to the concentration of 1.2 mM. However, at 1.5 mM caffeic acid, approximately 0.3 mM was not converted into EC, which suggested that *AtCAT1* could convert more than 1.2 mM (216.2 mg/L) caffeic acid and 1% ethanol (about 0.36 M) was not the limiting factor.

Synthesis of EC without feeding caffeic acid

We attempted to synthesize EC without feeding caffeic acid to *E. coli*. Caffeic acid was synthesized from tyrosine using *SeTAL* and *HpaBC* [27]. *SeTAL* converts tyrosine into *p*-coumaric acid, which is converted to caffeic acid by *HpaBC*. The two genes were introduced into *E. coli* harboring pC-OS4CL-AtCAT1. The resulting *E. coli* strain B-EC3 was fed ethanol thereafter. The culture filtrate from the strain B-EC3 showed a peak that had the same retention time as EC. Furthermore, no detectable caffeic acid was observed, indicating that all the synthesized caffeic acid had been converted to EC, and synthesis of more caffeic acid could increase the final titer of EC in *E. coli*. Therefore, we engineered *E. coli* to synthesize more tyrosine, which is a precursor of caffeic acid, by introducing genes, which are known to be

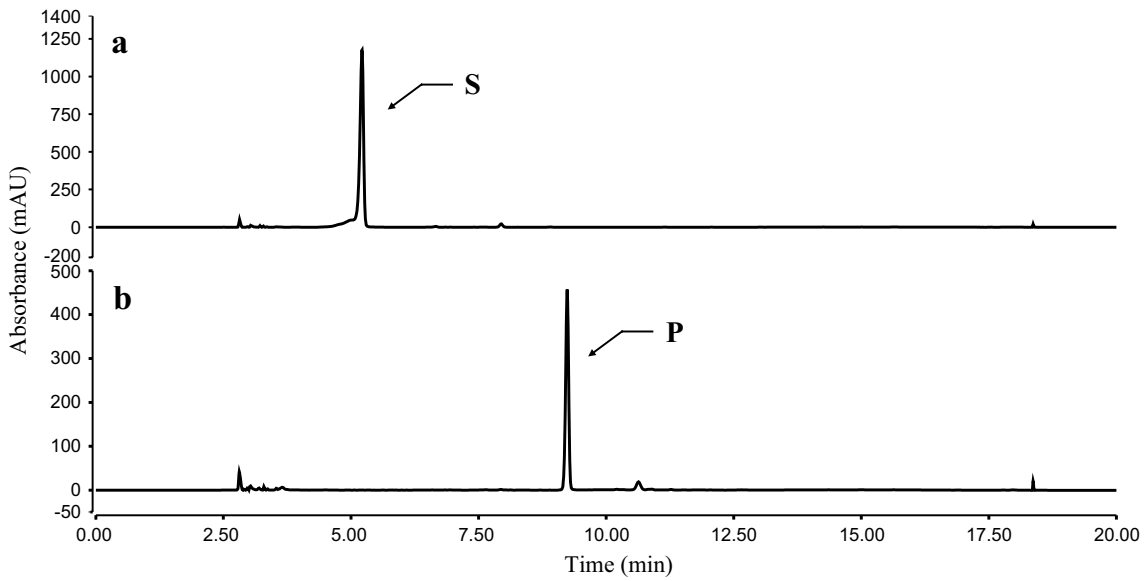

Fig. 2 High performance liquid chromatography (HPLC) analysis. **a** HPLC profile of standard caffeic acid (S); **b** HPLC profile of reaction product (P) from B-EC1 strain supplied with 100 μM caffeic acid. P was identified to be ethyl caffeate

involved in the synthesis of tyrosine in *E. coli*. We tested six constructs (pA-aroG-tyrA, pA-aroG^{f}-tyrA^{f}, pA-aroG^{f}-ppsA-tktA-tyrA^{f}, pA-aroL-aroG^{f}-ppsA-tktA-tyrA^{f}, pA-aroL-aroE-aroD-aroB-aroG^{f}-ppsA-tktA-tyrA^{f}, and pA-aroC-aroA-aroL-aroE-aroD-aroB-aroG^{f}-ppsA-tktA-tyrA^{f}) with various combination of genes relevant to the shikimate pathway of *E. coli*, including *tyrA, aroG, tyrA^{f}, aroG^{f}, aroC, aroA, aroL, aroE, aroD, aroB, ppsA*, and *tktA* [24, 28]. Each construct was transformed into *E. coli* strain B-EC3 and the resulting strains (B-EC4 – B-EC9) were tested for the synthesis of EC. The *E. coli* strains overexpressing any of these constructs showed better titer than B-EC3, which did not overexpress the shikimate pathway gene-module construct. The *E. coli* strain B-EC7, containing five genes, namely *aroL*, *aroG^{fbr}*, *ppsA*, *tktA*, and *tyrA^{fbr}*, produced the highest titer of EC (78.8 mg/L), followed by B-EC8 (68.6 mg/L), B-EC6 (68.5 mg/L), B-EC5 (65.2 mg/L), B-EC9 (59.1 mg/L), B-EC4 (44.8 mg/L), and B-EC3 (38.6 mg/L) (Fig. 3). This result agreed with the previous studies in which the overexpression of shikimate gene module increased the final titer of the synthesized compound; however, the best gene-module construct was different depending on the compound synthesized [24, 28, 29].

Our feeding study had shown that approximately 216.2 mg/L (1.2 mL) caffeic acid was converted to EC. The current EC titer was approximately 78.8 mg/L (0.38 mM), which indicated that the final titer could be increased further. In order to increase the final titer of EC, we used *E. coli* mutants with deletion of genes from the shikimate pathway, for further production of tyrosine. The BT strain, in which *tyrR* was deleted, and the BTP strain, in which *tyrR* and *tyrA* were deleted, were used to increase the production of tyrosine [21]. We introduced pC-OS4CL-AtCAT1 and pA-aroL-aroG^{fbr}-ppsA-tktA-tyrA^{fbr} into BT and BTP (BT-EC7, BTP-EC7) to examine the titer of EC. Both the mutant strains showed better titer than the wild type. The *E. coli* strain BT-EC7 synthesized approximately 116.7 mg/L EC, which was 1.48-fold higher than in the wild-type strain (Fig. 4).

EC is a natural compound found in some plants, and its biosynthetic pathway is not yet known. Here, we designed the biosynthetic pathway, assembled genes for the synthesis of EC, and successfully synthesized the compound. Rational design of the biosynthetic pathway and selection of genes from diverse sources can make the synthesis of diverse compounds feasible. Furthermore, this could lead to the synthesis of unnatural compounds, derived from natural compounds. For example, we could

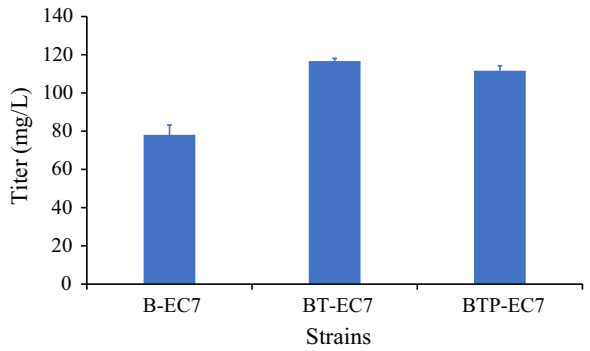

Fig. 4 Synthesis of ethyl caffeate in *E. coli* mutants expressing *Os4CL*, *AtCAT1*, *SeTAL HpaBC*, *aroL*, *aroG^{fbr}*, *ppsA*, *tktA*, and *tyrA^{fbr}*

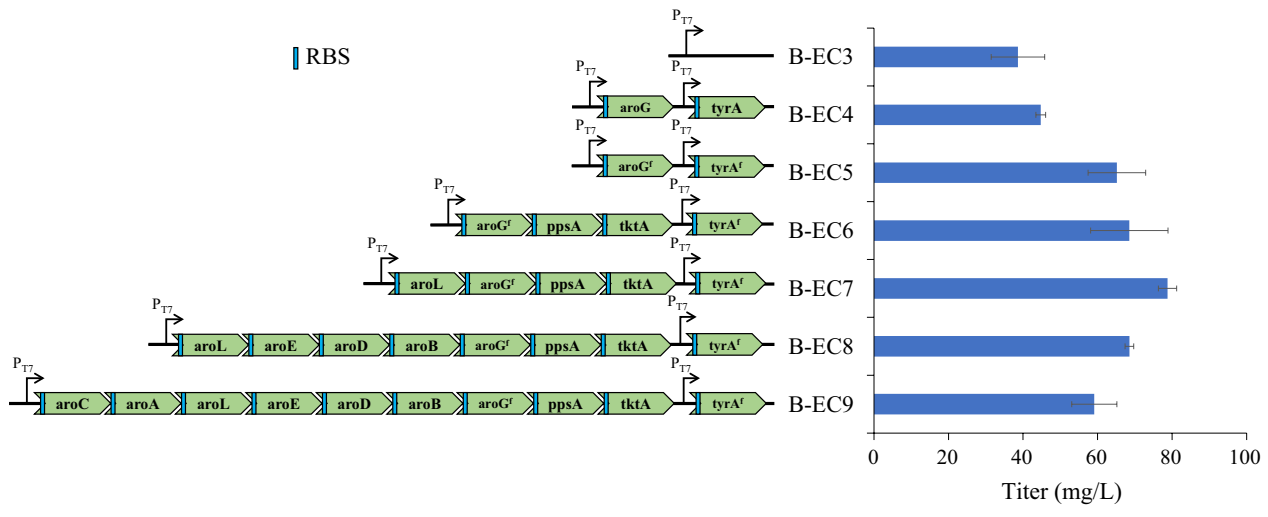

Fig. 3 Constructs containing shikimate pathway genes, used for the overproduction of tyrosine (left), and synthesis of ethyl caffeate in *E. coli* expressing corresponding constructs (right). All strains had genes *Os4CL*, *AtCAT1*, *SeTAL*, and *HpaBC*. P_{T7}, T7 promoter; RBS, ribosome-binding site

synthesize at least four other alkyl caffeates, depending on the alcohol, which have not been found in nature yet, and the compounds could potentially have different biological activities.

Authors' contributions
SWL and JHA designed the experiments. SWL and HK performed the experiments. SWL, HK, and JHA analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials
All data generated or analyzed during the present study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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